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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PS 2958 for a patent by UNIVERSITY OF TASMANIA as filed on 13 June 2002.

WITNESS my hand this
Twenty-fifth day of June 2003

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AUSTRALIA

Patents Act 1990

University of Tasmania

PROVISIONAL SPECIFICATION

Invention Title:

Metallothionein based neuronal therapeutic and therapeutic methods

The invention is described in the following statement:

Introduction to the Invention

This invention relates to the use of metallothionein as an active ingredient in effecting and enhancing recovery of damaged neuronal tissue, particularly following physical trauma and damage thereto. The invention provides a therapeutic incorporating metallothionein and methods of treatment based therefor.

Background to the Invention

Metallothionein (MT) is a naturally occurring peptide, which is present in most cells of the mammalian body. There are many isoforms in humans, but these resolve into four classes; MT-I and MT-II which are expressed widely, MT-III which is mainly found in the brain, and MT-IV which is restricted to specific epithelial sites. MTs are intracellular proteins with occasional nuclear localisation, and although there are persistent reports of extracellular detection of MT, the prevailing dogma is fixed that their physiological role is within cells.

MTs are metal binding proteins (61-67 amino acids), which normally bind seven zinc ions, although zinc/copper mixtures have been reported. Some isoforms are rapidly induced in response to increases in zinc or copper levels, and also by a large number of hormones and cytokines, including glucocorticoids, interleukin 1 and 6, interferons and so on. Their exact physiological role is unclear. Early suggestions that they act to prevent accumulation of toxic levels of heavy metals are no longer favoured, and if their role is indeed in metal metabolism, it is more likely that they are involved in the intracellular homeostasis of zinc. However, MTs are efficient scavengers of free radicals and are able to protect DNA and other molecules from oxidation, suggesting that their function may be protective. MTs may be considered intracellular stress proteins which respond to a wide variety of stimuli.

It is relevant that MT-I/II knockout animals, and those which overexpress MT-I and MT-II are phenotypically normal, except for sensitivity and resistance, respectively, to some chemical and physical stresses.

Deficiency of MT-III, the "brain-specific" class of MT, has been implicated in the pathogenesis of Alzheimer's disease, although this finding has been strongly disputed. MT-III reduces neuronal survival, and the applicants have shown it, when added to cultured neurons, reduces neurite sprouting. Exogenous MT-III appears to have an opposing effect to MT-IIA and it is

expected that comparison of their structures will reveal strategies for designing analogues of both which have specific neurotrophic properties. It has been shown that exposure of rat brain lesions to MT-III causes vacuolisation, consistent with extensive neuronal death.

Metallothionein and Heavy Metals

There is a large body of literature on the relationship between metallothionein and heavy metals, particularly cadmium. MT was originally isolated as a cadmium-binding protein, and it is known that it acts as the major intracellular sink for this toxic metal. Hence, people exposed to cadmium in the workplace or through contaminated diet will have elevated metallothionein levels, particularly in the kidney. There is no question that MT acts to protect cells against cadmium, however it is not an effective agent, nor is it likely that this is the actual physiological role for the protein: it is likely an adventitious property derived from the chemical similarity between zinc and cadmium. One consequence of this is the numerous studies of the pharmacokinetics of metallothionein bound to heavy metals, following various routes of administration. Whilst cadmiummetallothionein is (not surprisingly) toxic, it is not believed that naturally-occurring zinc or copper-metallothioneins, will have significant metal-based toxicity.

The applicants examined the action of metallothionein proteins; including MT-IIA, a major human metallothionein of the MT-I/II class. The studies found that administration of metallothionein to cultured neurons increases neuronal survival and enhances the rate of axonal extension. Furthermore, in lesioned rat brains, metallothionein enhances regenerative axonal extension into the lesions and replacement of damaged tissue. Accordingly, the use of metallothionein as an active ingredient in neuronal therapy provides a novel method of stimulating neuronal growth and neuronal survival, a novel class of therapeutic agents and a novel method of treatment for a range of neuronally based diseased states.

Moreover, metallothionein offers several practical advantages as a therapeutic agent.

1. It is a naturally occurring, non-toxic protein
2. It appears possible that intraperitoneally administered metallothionein can enter the CNS compartment, following physical trauma to the brain or spinal cord or breakdown of the blood-brain barrier due to other causes.

3. Metallothionein is not post-translationally modified and hence can be easily produced in bacterial or other expression systems
4. Metallothionein is a small peptide (61 amino acids) and it is very likely that a novel analogue which is amenable to chemical synthesis can be designed.

Statement of the Invention

In one aspect the invention provides a method of stimulating neuronal growth comprising contacting a target neuron with metallothionein.

The target neuron is preferably contacted by direct physical interaction with metallothionein.

The target neuron may have suffered physical trauma including lesion or other forms of neurodegeneration.

The metallothionein may be selected from any one or a combination of known metallothionein isoforms including MT-I, MT-II, MT-III and MT-IV.

Most preferably the metallothionein is selected from MT-II including human MT-IIA.

The metallothionein may be a synthetic analogue which combines structural or physical features of any or all known metallothionein isoforms.

The metallothionein may be provided in a concentration of about 5µg/ml.

The method of the invention may be applied to a range of compromised neuronal states including diseased states and injuries.

In another aspect the invention provides a method of treatment of any one or a combination of Alzheimers, Parkinsons, Motor Neuron Diseases, head injury, spinal cord trauma and glaucoma comprising the administration to a patient of a therapeutic including metallothionein as previously described as an active ingredient wherein said therapeutic is applied or administered so as to directly interact with the site of neuronal compromise.

In another aspect the invention provides a therapeutic composition comprising metallothionein in any one or a combination of isoforms, or as a synthetic metallothionein comprising features of one or more isoforms, as an active ingredient in a pharmaceutically acceptable carrier wherein said carrier is adapted for topical administration to an area of neuronal compromise.

The composition may be adapted for direct topical application to exposed neurons or for intraperitoneal administration to non-exposed neurons.

Detailed Description of the Invention

The invention will now be described with reference to particular embodiments and examples which are not limiting to the scope of the invention.

Evidence

The action of MT-IIA (a major human metallothionein of the MT-I/-II class) in two distinct culture models of rat neurons, and in a rat *in vivo* model of cortical damage was examined. In culture, it was found that administration of MT-IIA increases neuronal survival, and enhances the rate of axonal extension. In lesioned rat brains, it was found that MT-IIA enhances regenerative axonal extension into the lesion, and replacement of damaged tissue.

Metallothionein Action on Cultured Rat Cortical Neurons:

Rat cortical neurons (E18) were plated at low density in neurobasal medium + B27, including 150 μg/ml of a brain extract. Recombinant MT-IIA was produced (the major human metallothionein I/II isoform) in *E. coli* cultures and reconstituted it as a zincthionein (7 moles zinc/mole protein).

Figure 1 shows that:

(A) Human MT-IIA promotes neuron survival over 3 days [$p < 0.01$, ANOVA]. Hence, Zn-MT is not detrimental to the survival of cultured neurons.

(B) and (C) However, under the same conditions, human MT-IIA *does not* increase the initiation of new neurite sprouting over 3 days, expressed as either the percentage of neurite bearing neurons (B) or number of neurites per neuron(C) [$p > 0.01$, ANOVA]. This is important, because *inappropriate* sprouting of neurons has been associated with premature neuronal death.

(D) However, MT-IIA does dose-dependently promote neurite *elongation* during this period (D). [$p < 0.01$, ANOVA]

It was concluded from these experiments that MT-IIA is able to enhance neurite elongation in culture, without increasing the rate of neurite sprouting.

In the next series of experiments, neurons were maintained in culture for 21 days, to allow formation of clusters which are interconnected by fasciculated bundles of axons.

The axons were then cut with a microscapel and recombinant MT-IIA added.

Figure 2 shows that 12 hours after cutting the neuronal bundles, there is a marked retraction by the cut neurites. In the absence of MT-IIA, there are very few neurite extensions, as assessed by NF-M, Tau or β-tubulin immunohistochemistry (markers for axons). However, 12 hours of incubation with MT-IIA (5 μg/ml) the number and length of processes extending into the lesion site increase.

These experiments were repeated, but with a longer exposure (18 hours) to recombinant metallothionein:

Figure 3 shows that 18 hours after lesion, in vehicle-treated animals, there is some neurite growth into the lesion site, as assessed by immunohistochemical markers. However, in the presence of MT-IIA (5 μg/ml) the processes have completely traversed the lesion site.

It was concluded from these experiments that MT-IIA dramatically increases the extension of processes (most likely axons) between clusters, following lesion by microscalpel, and that after 18 hours of exposure, axonal bundles have reformed between the clusters. This effect of MT-IIA the result of a direct interaction between the protein, the neurons and the culture medium.

Metallothionein Action in a Rat Model of Cortical Injury

A rat model of physical damage to the cortex was developed, and the applicants have extensively characterised it in terms of neuronal damage, morphology, and subsequent recovery (cf publications, below). Interestingly, this model is not only representative of traumatic damage in humans, but has extensive similarities to pathological hallmarks of early Alzheimer's Disease. The effect of recombinant human MT-IIA at timepoints after initiation of injury was examined.

Figures 4 and 5 show the extent of the physical injury, and microglial invasion of the cavity. MT-IIA administration reduced microglial infiltration and promoted formation of a tissue bridge across the lesion, from the pial surface down. MT

also promoted axonal extension into the lesion site. Very few axonal extensions were seen in the control rats.

Figure 4 shows the global location of needle stick injuries is indicated in Panel A. Brain sections underwent immunohistochemistry against SMI-312 (green) and ferritin (red) 4 days post injury. Needle stick injury resulted in a large injury tract, and microglial migration into and surrounding the injury site (B). MT-IIA treatment reduced microglial infiltration, and promoted the formation of a tissue bridge enclosing the lesion site from the pial surface down, forming a teardrop like invagination (C). Microglia at the pial surface were small and round, in contrast to the large, amoeboid microglia observed in deeper cortical layers (D, E respectively). MT-IIA promoted regenerative axonal growth into the lesion site at both the pial layer (D) and deeper cortical layers (E). In contrast, very few axonal extensions were visualised in control rats, at the pial level (F) or deeper cortical layers (G). Arrowheads indicate the injury tract.

Figure 5 shows immunohistochemical staining against SMI-312 (green) and ferritin (red) 4 days post injury. SMI-312 immunoreactive axonal extensions were often found in close association with small round microglia at the pial surface (A, B). Contrastingly, axonal extensions in deeper cortical layers were often not associated with larger, amoeboid microglia (C, D). Regenerating axons often exhibited a wavy morphology, as if they were constantly changing direction. Occasionally, pyramidal (indicated by arrow, C) and bulb-like (indicated by arrow, D) accumulations were observed along axonal sprouts.

It was concluded from these exciting experiments that administration of recombinant MT-IIA has dramatically increased the rate of recovery from these physical injuries. Based on the culture experiments, it is proposed that administration of MT-IIA following CNS injury, acts directly on neurons to increase the rate of axonal extension into the lesion.

Some examples of clinical applications of the invention are detailed as follows:

Disease	Indication	Role of MTI/II (IIA)
Alzheimer's disease	Promote nerve cell survival. Promote neuronal regeneration. Buffer metals implicated in development of pathological hallmarks.	It was demonstrated that MTI/II is upregulated in early stages of the disease (published)
Parkinson's disease	Promote nerve cell survival. Promote regeneration. Buffer metals implicated in toxicity.	Evidence of abnormal metal homeostasis in the brain as well as neurodegeneration.
Motor neuron disease	Promote nerve cell survival. Promote neuronal regeneration. Buffer metals implicated in toxicity. Reduce oxidative stress implicated in neuronal degeneration.	Evidence of abnormal metal homeostasis in the brain and spinal cord as well as neurodegeneration.
Head injury	Promote nerve cell survival. Promote neuronal regeneration.	It was shown that MT I/II is upregulated at zone of injury. Recombinant protein promotes brain healing and axonal regeneration
Spinal cord trauma	Promote nerve cell survival. Promote neuronal regeneration.	Evidence of delayed neurodegeneration and spinal cavitation following injury. The recombinant protein is potentially capable of promoting neural healing and regeneration.
Glaucoma	Promote nerve cell survival. Promote neuronal regeneration.	Axonal damage followed by neurodegeneration underlies the disease. MTI/II may potentially promote survival of nerve cells and/or appropriate regeneration.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the

specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this thirteenth day of June 2002

University of Tasmania
Patent Attorneys for the Applicant:

F B RICE & CO

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Figure 1

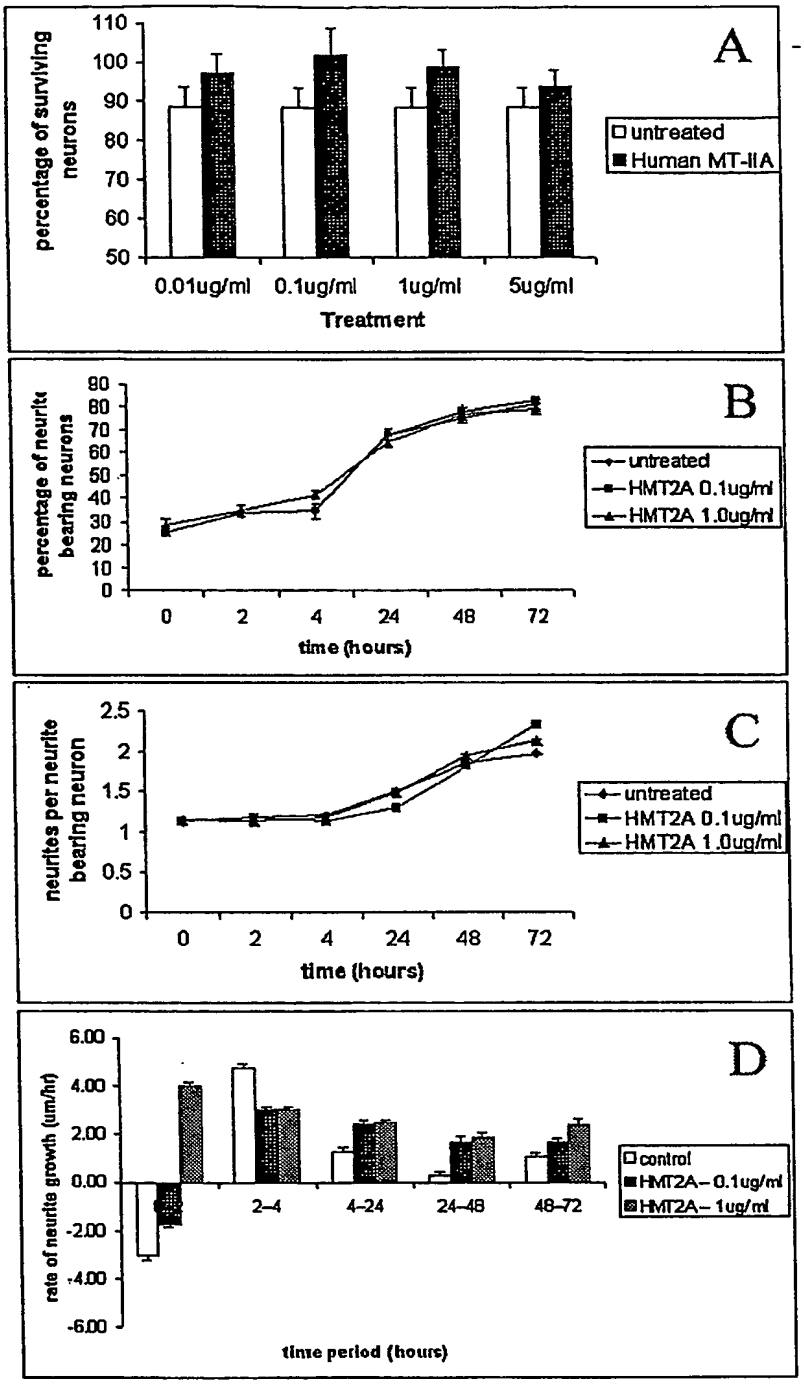


Figure 1: Human MT-IIA promotes neuron survival in the presence of adult rat brain extract (150µg/ml) after 3 days (A). [$p < 0.01$, ANOVA] However, under the same conditions, human MT-IIA has no effect on neurite sprouting over 3 days, in either the percentage of neurite bearing neurons (B) or the number of neurites per neuron (C). [$p > 0.01$, ANOVA] MT-IIA dose dependently promoted neurite elongation during this period (D). [$p < 0.01$, ANOVA] For all graphs, error bars represent standard error values.

Figure 2

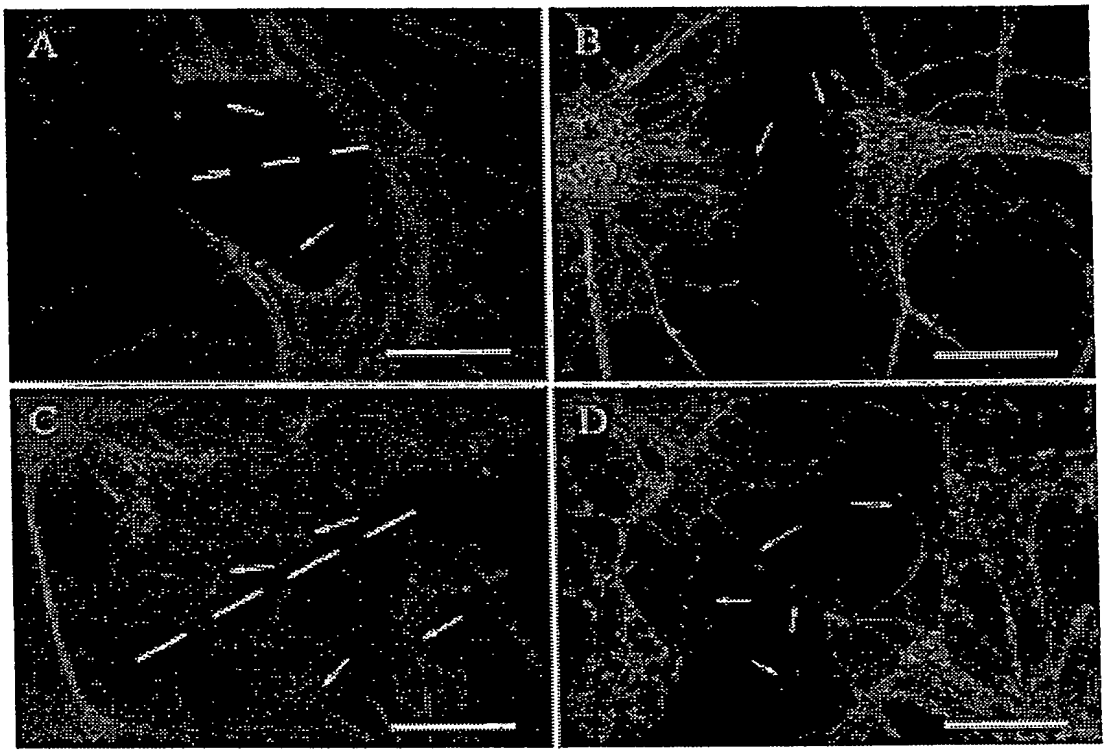


Figure 2: At 12 hours post injury, there is a marked retraction by transected neurites from the lesion site (indicated by the broken line) of up to 100μm. Whilst there were very few NF-M immunoreactive processes (red) extending into the area of retraction (indicated by arrows) in untreated neurons (A, scale bar = 100μm) there were many in MT-IIA treated neurons (C, scale bar = 200μm). Tau (red) and β-tubulin (green) immunohistochemical analysis also indicates very few processes extended into the area of retraction (indicated by arrows)(B, scale bar = 100μm). In the presence of MT-IIA, these processes are significantly longer (D, scale bar = 50μm).

Figure 3

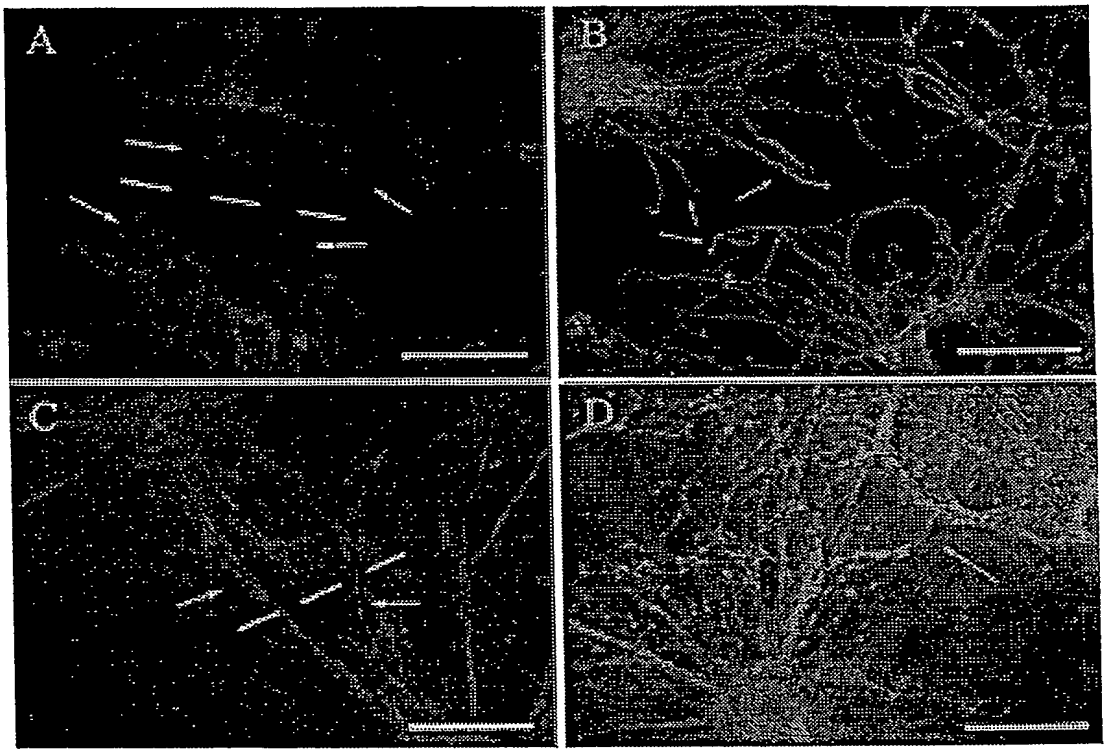


Figure 3: NF-M immunohistochemical analysis of neurite transections 18 hours post injury, both untreated (A, scale bar = 100μm) and MT-IIA treated (C, scale bar = 100μm;). NF-M immunoreactive processes (red) extend from the neurite stumps (indicated by arrows) and grow towards the central lesion site (indicated by the broken line). MT-IIA promotes growth of NF-M immunoreactive processes (indicated by arrows) across the central lesion site (C). Tau (red) and β-tubulin (green) immunohistochemical analysis also indicates a number of processes extended into the area of retraction (indicated by arrows) in untreated neurons, but again these processes do not cross the transection site (B, scale bar = 50μm). MT-IIA promotes the growth of processes across the transection site (indicated by arrows) and to the opposite stump of the transected neurite bundle (D, scale bar = 100μm).

Figure 4

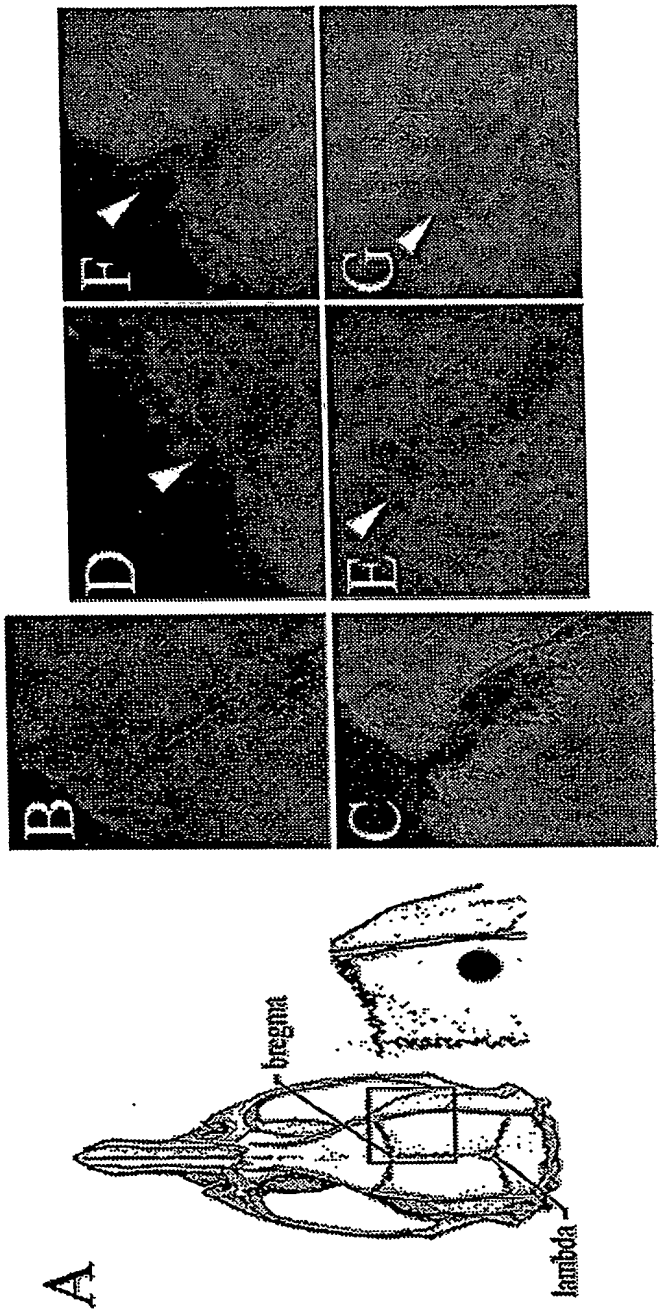
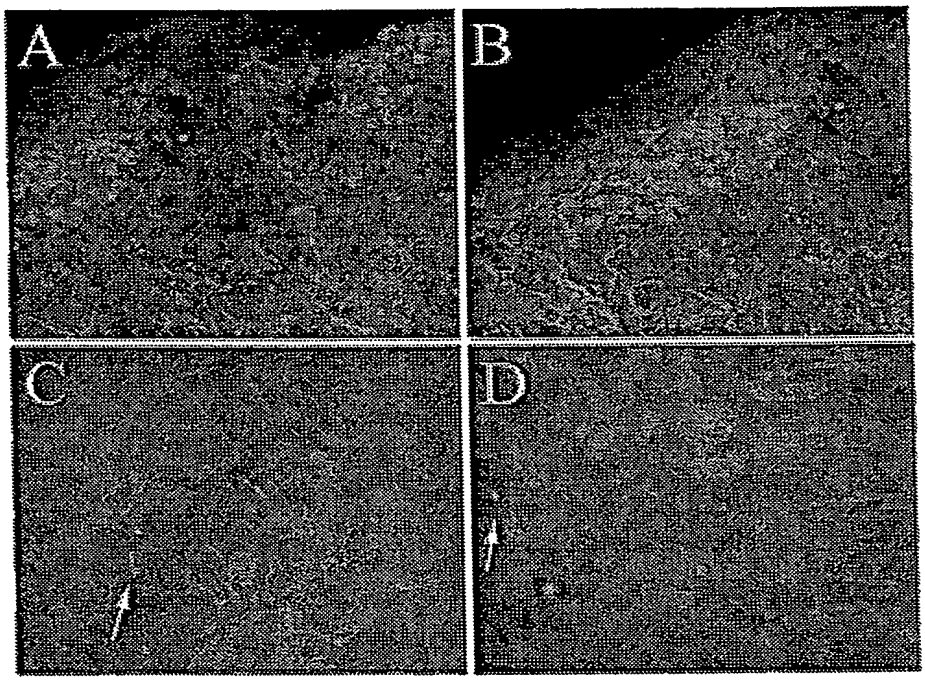


Figure 5



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